BIOACCUMULATION OF PHARMACEUTICALS IN FISH AS PART OF ENVIRONMENTAL RISK ASSESSMENT OF HUMAN MEDICINES

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Tiivistelmä/Referat – Abstract

Pharmaceutical contaminants in waste and surface waters have been recognized as an emerging risk to environmental health. Bioaccumulation of pharmaceuticals increases the risk of adverse effects in off-target species, as the chemical concentration within the organism exceeds the concentration of the surrounding environment. An organism's ability to metabolize foreign organic compounds influences the likelihood of bioaccumulation. Current methods for predicting bioaccumulation in aquatic organisms are labour intensive or too simplistic to cover the variety of chemical and physiological processes involved and may lead to over or underestimations of environmental risk. A promising approach to improve bioaccumulation predictions, without the need of excessive animal testing, is to incorporate *in vitro* biotransformation data into computational models.

The primary aim of this study was to assess whether selected pharmaceuticals (diclofenac, gemfibrozil, haloperidol, levomepromazine, levonorgestrel, sertraline and risperidone), that are well metabolized in humans through key biotransformation pathways, are metabolized by rainbow trout (*Oncorhynchus mykiss*) liver enzymes under physiologically relevant conditions (11°C, pH 7.8). A secondary aim was to produce fish *in vitro* intrinsic clearance ($CL_{int,\ in\ vitro}$) data, that could potentially be used as input in computational models to predict bioaccumulation.

In vitro biotransformation was studied using a single vial approach according to the Organisation for Economic Co-operation and Development (OECD) Test Guideline 319B: *Determination of in vitro intrinsic clearance using rainbow trout liver S9 sub-cellular fraction (RT-S9).* Depletion of the test compounds were measured during a 3-hour incubation period. High-performance liquid chromatography with ultraviolet detection (HPLC–UV) was used for qualitative and quantitative analysis of the samples.

Levomepromazine, levonorgestrel and sertraline showed significant substrate depletion compared to negative controls while gemfibrozil, haloperidol, and risperidone did not seem to be metabolized. The results for verapamil were inconclusive. Levomepromazine displayed a higher *in vitro* intrinsic clearance rate (26 ml/h/g liver) than diclofenac (6.2 ml/h/g liver).

These results are in accordance with previous studies and support the notion that a direct comparability between fish and human metabolism cannot be assumed, highlighting the need of fish *in vitro* biotransformation studies. The apparent lack of *in vitro* metabolism of risperidone, haloperidol, and gemfibrozil combined with their lipophilicity suggest that they are more likely to accumulate within rainbow trout, compared with the compounds that showed depletion during the assays, although repetitions and additional studies are needed to confirm this.

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bioaccumulation, pharmaceuticals in the environment, rainbow trout, *in vitro* intrinsic clearance, levomepromazine

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Tiivistelmä/Referat – Abstract

Vesiympäristössä esiintyy lääkeainejäämiä, joista osa voi kertyä kaloihin ja muihin vesieliöihin, jolloin eliössä oleva lääkeainepitoisuus voi nousta moninkertaiseksi ympäröivään vesistöön verrattuna. Biokertyvyys lisää luonnossa ilmenevien haittavaikutusten todennäköisyyttä ja sen määrittäminen on siksi tärkeä osa lääkeaineiden ympäristöriskin arviointia. Tällä hetkellä käytössä olevat, biokertyvyyttä ennustavat menetelmät perustuvat joko laajoihin eläinkokeisiin, tai ovat hyvin yksinkertaistettuja eivätkä ota huomioon oleellisia biologisia prosesseja, mikä voi johtaa ympäristöriskin yli- tai aliarviointiin. Eliön kyky metaboloida lääkeainetta pienentää biokertyvyyden todennäköisyyttä tehostamalla lääkeaineen poistumista elimistöstä. Metaboliaa voidaan mitata *in vitro* menetelmin, ja näin saadun datan integroiminen osaksi laskennallisia malleja onkin osoitettu parantavan mallien kykyä ennustaa biokertymistä.

Tutkimuksen päätavoitteena oli selvittää valikoitujen, ihmisessä metaboloituvien, lääkeaineiden (diklofenaakki, gemfibrotsiili, haloperidoli, levomepromatsiini, levonorgestreeli, sertraliini, risperidoni) metaboliaa kalassa, käyttäen mallina kirjolohen (*Oncorhynchus mykiss*) maksan S9-fraktiota. Toissijaisena tavoitteena oli tuottaa kvantitatiivista dataa, jota voitaisiin hyödyntää biokertyvyyttä ennustavissa laskennallisissa malleissa.

In vitro metaboliaa tutkittiin Taloudellisen yhteistyön ja kehityksen järjestön (OECD) laatiman ohjeen 319B: *Determination of in vitro intrinsic clearance using rainbow trout liver S9 sub-cellular fraction (RT-S9)* mukaan. Lääkeaineiden eliminaatiota mitattiin fysiologisissa olosuhteissa (11°C, pH 7.8), 3 tunnin inkubaatiojakson ajan. *In vitro* ominaispuhdistuma ($CL_{int,\ in\ vitro}$) määriteltiin diklofenaakille ja levomepromatsiinille. Näytteiden kvalitatiiviseen ja kvantitatiiviseen analyysiin käytettiin ultraviolettidetektorilla varustettua korkean erotuskyvyn nestekromatografiaa (HPLC-UV).

Levomepromatsiini, levonorgestreeli ja sertraliinin pitoisuudet laskivat merkittävästi kokeen aikana verrattuna negatiivisiin kontrolleihin, kun taas gemfibrotsiili, haloperidolin ja risperidonin pitoisuudet pysyivät muuttumattomina. Verapamiilin tulokset olivat epäselviä. Levomepromatsiinin eliminoitui nopeammin ($CL_{int,\ in\ vitro}$ = 26 ml/h/g maksaa) kuin diklofenaakki ($CL_{int,\ in\ vitro}$ = 6.2 ml/h/g maksaa).

Gemfibrotsiilin, haloperidolin ja risperidonin näennäisen metabolian puute yhdistettynä niiden lipofiilisyyteen viittaa siihen, että nämä lääkeaineet kertyvät kirjoloheen todennäköisemmin kuin ne lääkeaineet, joiden kohdalla havaittiin eliminaatiota. Toistoja sekä lisätutkimuksia kuitenkin tarvitaan näiden havaintojen vahvistamiseksi.

Avainsanat – Nyckelord – Keywords
biokertyminen, ympäristöriskin arviointi, lääkeainemetabolia, kirjolohi, maksan S9-fraktio, levomepromatsiini

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ABBREVIATIONS

API	Active pharmaceutical ingredient
ACN	Acetonitrile
BAF	Bioaccumulation factor
BCF	Bioconcentration factor
$CL_{int,\ in\ vitro}$	*In vitro* intrinsic clearance
CYP	Cytochrome P450
EMA	European Medicines Agency
ERA	Environmental risk assessment
GSH	Glutathione
GST	Glutathione S-transferase
HPLC	High Performance Liquid Chromatography
k_e	First-order depletion rate constant
K_{ow}	N-octanol/water partition coefficient, sometimes referred to as “P” in literature
LLOQ	The lower limit of quantitation
MeOH	Methanol
NADPH	β-Nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt hydrate
NSAID	Nonsteroidal anti-inflammatory drugs
OECD	Organisation for Economic Co-operation and Development
PAPS	3'-phosphoadenosine 5'-phosphosulfate
PEC	Predicted environmental concentration
PNEC	Predicted-no-effect-concentration
PBT	Persistent, bioaccumulative and toxic
QSAR	Quantitative structure-activity relationship
RT-S9	Rainbow trout liver S9 subcellular fraction
SULT	Sulfotransferase
UDPGA	Uridine-5'-diphosphoglucuronic acid trisodium salt
UGT	Uridine 5'-diphospho-glucuronosyltransferase
WWTP	Wastewater treatment plant

1 INTRODUCTION

Pharmaceutical contaminants in waste and surface waters have been recognized as an emerging risk to environmental and human health (SCHEER 2018). Studies show that some pharmaceuticals have a direct effect on aquatic wildlife at environmentally relevant concentrations, resulting in behavioural and physical changes impacting the survival of exposed populations (Kidd et al 2007; Brodin et al. 2013; Dzieweczynski et al. 2016). Pharmaceutical pollution also contributes to the development of antimicrobial resistance, amplifying the need of urgent and global action (Reinthaler et al.2003).

In recent years, great effort has been made to increase our understanding of the severity of the environmental impact of pharmaceuticals (European Commission 2019; European Commission 2020). While new information is constantly gathered, there is still a lack of knowledge in many areas, and further efforts must be made to improve the efficiency of the research by exploring new methods and updating current guidelines. One area of importance is the understanding of how pharmaceuticals accumulate in aquatic organisms. Bioaccumulation increases the risk of adverse effects in off-target species, as the chemical concentration within the organism exceeds the concentration of the surrounding environment. Even a slight increase in exposure to a pharmaceutical substance might be critical, especially for sensitive species like fish, as pharmaceuticals are designed to have biological activity at low concentrations. Although the pharmacokinetic properties of pharmaceuticals in human are routinely studied as a part of the drug-development process, uptake and elimination of pharmaceuticals in fish is less well understood.

To grasp the challenge of pharmaceuticals in the environment is a hard task due to its comprehensiveness and complexity. In efforts to give an overview of the named challenge, different areas that are of relevance in understanding why and how pharmaceuticals pose a risk to the natural environment is covered in the first part of this thesis. This is followed by a description of the environmental risk assessment process and an overview of bioaccumulation, and how it is currently evaluated. The focus is on small molecule drugs intended for human use. Biopharmaceuticals and medicines used in veterinary care are outside the scope of this thesis.

2 PHARMACEUTICALS AS ENVIRONMENTAL CONTAMINANTS

2.1 Emission sources

Pharmaceutical contaminants can enter the environment throughout a drug's life cycle, from manufacturing to use and disposal (aus der Beek et al. 2016). The contaminant can be the active pharmaceutical ingredient (API) itself, a metabolite, or other transformation product produced by biotic or abiotic modification of the parent compound. There are three main pathways by which pharmaceutical contaminants reach the environment: through the use of medicines, wastewater discharge from manufacturing sites, and improper disposal of unused pharmaceutical products. Regular use of medicines is considered the most important source of pharmaceutical contaminants into the environment globally, estimated to be 88% of total emissions, whereas discharge from manufacturing sites and improper disposal of drugs can have a significant local impact (Schwarzbauer et al. 2002; Fick et al. 2009; AstraZeneca 2018).

To understand why most of the pharmaceutical contaminants that end up in the environment originate from regular use of medicines, we need to understand what happens to APIs after administration. After the administration of a pharmaceutical product, the API is absorbed and distributed in the body before it is eliminated. Elimination occurs through metabolism, direct excretion, or a combination of the two. Drug metabolism is the process in which metabolic enzymes modify the drug, usually to a more hydrophilic metabolite in order to speed up the excretion process. The drug is therefore eventually excreted from the body in its original form or as metabolites via urine or faeces. As a result, APIs and their metabolites end up in municipal wastewaters and eventually find their way through the sewage systems to wastewater treatment plants (WWTP).

When pharmaceutical contaminants are exposed to wastewater treatment processes they can transform, degrade, adsorb to sludge, or pass through unchanged (Miège et al. 2009; Jelic et al. 2011). To measure how well treatment processes can remove pharmaceutical contaminants from wastewater, a removal rate is calculated, by comparing the concentration of the API in the water entering the treatment plant to the API concentration

of outflowing water (UNESCO and HELCOM 2017). These aquatic removal rates vary greatly between pharmaceuticals and different treatment facilities and should not be interpreted as direct measurement of total environmental burden since they do not account for active metabolites or alternative exposure routes besides effluent discharge from WWTPs. Even so, most APIs are incompletely removed from wastewater which contributes to WWTP effluents being the primary outlet of pharmaceuticals into the environment. As modern medicine relies on the use of medicinal products and drug consumption has been steadily increasing the last two decades, pharmaceutical contaminants will inevitably end up in the environment until more effective, affordable removal methods have been developed and widely introduced (OECD 2021).

2.2 Environmental fate

Pharmaceuticals have been found all around the world in a wide variety of environmental compartments including nontarget organisms (aus der Beek et al. 2016; UNESCO and HELCOM 2017). Surface water is the most studied medium and pharmaceuticals are typically found in the environment at trace amounts, with the measured concentrations ranging from nanograms to a few micrograms per litre. The environmental fate of pharmaceuticals, i.e. how they behave in the environment, is influenced by both intrinsic properties of the compound, such as water solubility, lipophilicity, and other physicochemical properties, as well as external factors, such as pH and sunlight, making the link between consumption and environmental occurrence less straight forward (Yamamoto et al. 2009). Even though the environmental fate of pharmaceuticals is hard to predict, some insight on how APIs interact with the environment can be gained by studying their physicochemical properties. Although the physicochemical properties of different API’s vary greatly, even within the same therapeutic group, there are some common denominators that can be used to describe pharmaceuticals in general. These properties have an impact on the absorption, metabolism, distribution, and excretion of the pharmaceutical substance and are therefore applied with selection pressure during the drug discovery and development process.

Pharmaceuticals are mainly distributed throughout the environment within bodies of water and through food chains as they are designed to have aqueous solubility and properties that allow for membrane permeability to optimize absorption from the gastrointestinal tract (Martinez and Amidon 2002). Lipophilicity impacts membrane permeability, and most pharmaceuticals have a predicted n-octanol/water partition coefficient ($\log K_{ow}$) between 0 and 5 (DrugBank 2020, www.drugbank.ca). Increasing lipophilicity also increases the likelihood of compounds accumulating within non-target organisms (Arnot and Gobas 2006). To achieve water solubility, API's often contain polar and ionizable functional groups, which typically increase their reactivity and affect interactions with other compounds (Wenlock et al. 2003). Solubility and partitioning properties of API's may change significantly depending on the state of ionization, which in turn is dependent on the pH of the surrounding medium, making the predictions of environmental fate even more difficult. In addition, pharmaceuticals are relatively stable since they are optimized to sustain long shelf life and degradation in the human body, until they reach their target. The majority (62%) of pharmaceuticals that were listed in the fass.se database in 2018 and assessed for biodegradation, were classified as potentially persistent in the environment (Graae et al. 2019). Persistent compounds have an increased potential to travel far from their emission sites, accumulate, and cause chronic adverse effects in nontarget species.

2.3 Pharmacology and ecotoxicity

Pharmaceuticals are biologically active compounds, designed to have physiological effects at low concentrations (Benner and Stevens 2018). APIs interact with endogenous molecules, often proteins, which can be referred to as drug targets. The biochemical interaction between the drug and its target that results in a biological response is the drugs mechanism of action. Drugs are optimized to cause a selective biological response by improving the structural specificity of the compound to minimize possible interaction with off-target molecules. This is done to reduce possible adverse effects mediated by alternative mechanisms. In addition to assessing the pharmacodynamic properties, acute and chronic toxicity of the drug candidate is thoroughly studied during drug development.

The relative safety of a drug can be expressed with a therapeutic index, which is a quantitative expression of the relationship between the efficacy and toxicity of the drug. A high therapeutic index is preferable, since it indicates that the dose needed to cause a toxic effect is magnitudes higher than the therapeutic dose. However, the ratio varies greatly between pharmaceuticals and low therapeutic indices are allowed for pharmaceuticals used to treat difficult diseases. This is to say that pharmaceuticals differ in regard of pharmacological effect and toxicity profiles, which is of relevance when evaluating the environmental risk of individual APIs (Gunnarsson et al. 2019).

When studying ecotoxicity of pharmaceuticals, one of the subjects of interest is whether orthologs of human drug targets can be found in nontarget species. The term ortholog is used to describe similar biological structures or sequences found in different taxa that are derived from a common ancestor through speciation (Gabaldón and Koonin 2013). Because pharmaceuticals are found in the environment at low concentrations, where they may be biologically active but not toxic, it is reasonable to assume that possible adverse effects in nontarget species are more likely to occur through specific biochemical interactions between the drug and a target protein rather than by a non-specific mode of action like narcosis, especially in species that express drug target orthologs. Due to bioinformatics and large biological databases currently available, it is possible to screen for the presence of human drug target orthologs in off-target species by comparing genomes or protein sequence data (Verbruggen et al. 2018). Gunnarsson et al. (2008) used orthology prediction to map the presence of human drug target orthologs in species commonly used as animal models in ecotoxicity assessments. Fish and frog were predicted to express the greatest number of orthologs with the highest degree of similarity, making them overall more sensitive to pharmaceuticals in the environment compared to more evolutionarily distant species. Drugs that target evolutionarily well conserved proteins potentially pose a greater risk for organisms in general. Even though the absence of drug target orthologs does not exclude the possibility of specific protein interaction, as well as the presence of target orthologs does not guarantee it, the research group argues convincingly by reflecting their findings against empirical data, that target orthologs are a matter of importance when evaluating the ecotoxicological risk of pharmaceuticals.

3 ENVIRONMENTAL RISK ASSESSMENT OF PHARMACEUTICALS

Environmental risk can be defined as a combination of the hazardous properties of a compound and its environmental exposure (European Chemicals Agency 2011). Whether a compound is considered hazardous, is based on its inherent properties such as persistency, bioaccumulation potential, and toxicity in the environment. This means that a compound can be highly hazardous without posing a risk for the environment if exposure is eliminated, while a less hazardous compound can pose a significant environmental risk if the emission volumes are high. Arguably all pharmaceutical substances have the potential to be harmful to the environment since they are biologically active and have the potential to reach the environment. However, to make a full environmental risk assessment on all pharmaceutical substances would be extremely expensive and time-consuming. Therefore, prioritisation schemes and trigger values are used in order to evaluate and categorize APIs based on how likely they are to cause environmental harm, with the aim of directing further research and mitigation efforts where it is needed the most (Burns et al. 2018).

3.1 Environmental risk assessment of new medicinal products for human use

Pharmaceutical companies are required to include an environmental risk assessment (ERA), performed according to guidelines provided by the European Medicine Agency (EMA), as a part of all new marketing authorisation applications since 2005 (Directive 2001/83/EC, 2001). The ERA is usually done in the end of the drug development process, along phase III clinical trials, and marketing authorisation for pharmaceuticals intended for human use cannot be denied based on environmental concerns (European Medicines Agency 2006).

The guideline on the environmental risk assessment of medicinal products for human use follows a tiered approach (Table 1), where an initial screening phase (phase I) is used to identify potential high-risk compounds (European Medicines Agency 2006). Trigger values for predicted environmental concentration in surface waters (PECsw) and n-

octanol/water partition coefficient ($logK_{ow}$) are used to determine whether a significant risk can be anticipated, and further tests are needed. PECsw is calculated based on the maximum daily dose consumed per inhabitant and a default or refined market penetration factor according to Figure 1. A PECsw value of 0.01 µg/l or higher, initiate phase 2 experimental studies for evaluation of aquatic effect, by determining a predicted-no-effect-concentration (PNEC), and environmental fate. If $logK_{ow}$ exceeds 4.5, persistence, bioaccumulation, and toxicity (PBT) are to be further studied according to REACH 'Guidance on information requirements and chemical safety assessment' (European Medicines Agency 2016a). If the possibility of environmental risk cannot be excluded after completion of phase 2 and PBT studies, this shall be indicated in the labelling of the product. Substances that are unlikely to cause significant risk (e.g. vitamins) can be exempted from ERA altogether, while some pharmaceuticals with specific mechanisms of actions are not included in phase 1 evaluation but undergo a tailored risk assessment. The latter applies mainly to hormones as they can be suspected to affect the reproduction of off-target species at concentrations below 0.01 µg/l. The guideline document is under revision, and some changes regarding which compounds require a tailored risk assessment is to be expected (European Medicines Agency 2016b).

Table 1. Features of different stages in the environmental risk assessment of pharmaceuticals (European Medicines Agency 2006)

Stage in regulatory evaluation	**Stage in risk assessment**	**Objective**	**Method**	**TEST / DATA REQUIREMENT**
Phase I	Pre-screening	Estimation of exposure	Action limit	Consumption data, logKow.
Phase II Tier A	Screening	Initial prediction of risk	Risk Assessment	Base set aquatic toxicology and fate
Phase II Tier B	Extended	Substance and compartment-specific refinement and risk assessment	Risk Assessment	Extended data set on emission, fate and effects

$$PEC_{SURFACEWATER} = \frac{DOSEai * Fpen}{WASTEWinhab * DILUTION}$$

Parameter	Symbol	Value	Unit	Origin	Remarks
Input					
• Maximum daily dose consumed per inhabitant	DOSEai		[mg·inh^{-1}·d^{-1}]	A	The highest recommended dose should be used
• Fraction of market penetration	Fpen	0.01	[--]	D	Default
• Amount of wastewater per inhabitant per day	*WASTEWinhab*	200	[L·inh^{-1}·d^{-1}]	D	
• Dilution factor	*DILUTION*	10	[--]	D	
Output	$PEC_{SURFACEWATER}$		[mg·L^{-1}]	O	
• Local surface water concentration					

A = information from Applicant, D = Default value, O = Output

Figure 1. The equation for calculating predicted environmental concentration (PEC) in surface water and a table with the default values used in the equation. Modified from 'Guideline on the environmental risk assessment of medicinal products for human use' (European Medicines Agency 2006).

3.2 Knowledge gaps and challenges

Even though environmental risk assessment is required as a part of market authorization in Europe, publicly available information on the environmental risk of pharmaceuticals is lacking, as data sharing is not mandatory due to legislative data protection rules (European Medicines Agency 2016b). More importantly, the majority of pharmaceuticals currently in use have been registered before 2006 and relevant environmental studies have not been performed or reported for most of these compounds (Burns et al. 2018; Gunnarsson et al. 2019). Efforts have been made to increase the availability, transparency, and extent of environmental information concerning pharmaceuticals (fass.se 2012, www.fass.se; Roos et al. 2012). These strategies include the collection and review of data provided voluntarily by the pharmaceutical industry as well as the development of new assessment and prioritisation tools.

The main challenge is to find an initial assessment method that can correctly identify all pharmaceuticals that are harmful to the environment while minimizing the number of false positives, without the need of extensive added research. To find a method that matches this ideal is not easy, as pharmaceuticals are such a large and heterogenous group of compounds in the regard of both physicochemical and pharmacodynamic properties. The most promising methods according to Roos et al. (2012) are risk-based, counting for both exposure and effect by including some form of PEC and PNEC or other predicted "no-effect" value. The octanol-water partition coefficient of the compound is also incorporated in commonly used risk assessment methods as a measure of lipophilicity to account for the compound's ability to cross biological membranes and enter organisms. It can be used as a part of risk calculations along with exposure and effect data, as in the fish plasma model described by Huggett et al. (2003), or as a trigger value on its own, as in phase 1 ERA of pharmaceuticals (European Medicines Agency 2006**)**. While lipophilicity is an important parameter that generally correlates with bioaccumulation potential and environmental toxicity, the K_{ow} is a highly simplified measure compared to the complex phenomena it is meant to represent, especially when the aim is to assess polar, ionizable compounds, and may therefore lead to over or underestimation of a compound's environmental risk (Meylan et al. 1999; Vestel et al. 2016; Armitage et al. 2017).

4 BIOACCUMULATION

Bioaccumulation is the process in which a chemical gradually accumulates in an organism, with its internal concentration eventually exceeding that of the surrounding environment. Elevated concentrations of pharmaceutical substances within organisms naturally increases the risk of adverse effects and toxicity. Even a slight increase of internal concentration might be critical, especially for sensitive species such as fish, that express numerous drug target orthologs. Bioaccumulation of chemicals can cause unpredictable effects on the individual, population and ecosystem level and should

therefore be viewed as a hazard criterion in itself (Tillitt et al., 1992; Franke et al. 1994). However, there is no scientific definition for a “bioaccumulative compound”, and the criterion, as well as the assessment methods, can therefore vary depending on which guideline is referred to (Gobas et al. 2009). In the following sections, the two main processes which influence accumulation of chemicals in biota, namely uptake and elimination, are briefly introduced before exploring how the bioaccumulation potential of pharmaceuticals is currently studied.

4.1 Uptake and elimination

Bioaccumulation includes both the direct uptake of chemicals via respiratory and dermal surfaces (bioconcentration) as well as uptake via contaminated food (biomagnification) (European Chemicals Agency 2011). When studying bioaccumulation of pharmaceuticals in fish both dietary and non-dietary routes can be of importance, whereas non-aquatic organisms are mainly exposed to pharmaceutical contaminants through diet (Park et al. 2009; Ruhí et al. 2016). While both bioconcentration and biomagnification are essential when evaluating the overall environmental impact of pharmaceuticals, the current methods for evaluating an APIs bioaccumulation potential are based on bioconcentration models, so they will be the focus of this thesis.

The amount of a chemical present in the environment that is accessible for direct uptake from water is influenced by both intrinsic properties of the compound as well as environmental factors (described previously in section 2.2). Generally, persistent lipophilic compounds that are freely dissolved in the aquatic environment are prone to enter and concentrate within fish and other aquatic organisms, as they are able to pass biological membranes by passive diffusion. Bioaccumulation occur when the rate of uptake exceeds the combined clearance rate of all the different elimination processes of the organism. Major routes of uptake and elimination of contaminants in fish are shown in Figure 2. Generally, a chemical is considered eliminated when it is no longer present in the organism in its original form**.** The main elimination processes in fish are passive diffusion across gill surfaces, fecal egestion, and biotransformation (Arnot and Gobas

2006). Biotransformation is an especially important elimination pathway for lipophilic chemicals, which are slow to be excreted if unmodified.

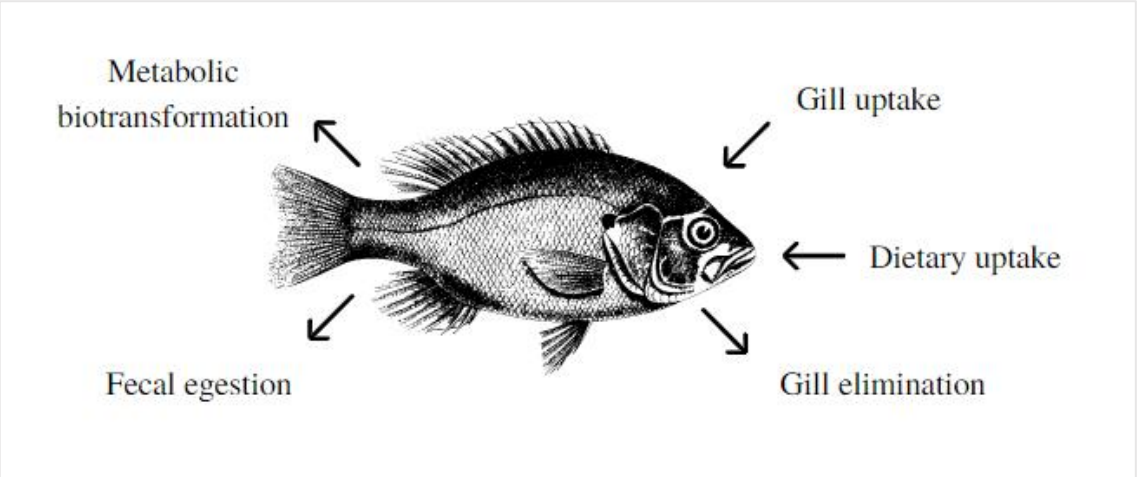

Figure 2. Major routes of uptake and elimination of contaminants for fish. Adapted from Arnot and Gobas (2006).

Like humans, fish are capable of metabolizing foreign organic compounds to more water-soluble metabolites and orthologs of enzymes responsible for biotransformation of pharmaceuticals in humans are present in fish (Chambers and Yarbrough 1976; González 2009; Goldstone 2010). Drug metabolism can be divided into Phase I and Phase II enzymatic reactions (Xu et. al. 2005). Phase I reactions include hydrolysis, reduction, and oxidation of the substrate and result in addition or exposure of reactive functional groups. Phase II reactions are conjugation reactions where a polar endogenous molecule (e.g. glucuronide, sulphate, and glutathione) is linked to the substrate. Often conjugation occur after Phase I reactions, but direct conjugation to APIs is also possible. The enzymes responsible for Phase I metabolism of most APIs belong to the superfamily of cytochrome P450 (CYP) while conjugation reactions are catalysed by transferases including uridine 5'-diphospho-glucuronosyltransferases (UGT), sulfotransferases (SULTs), and glutathione S-transferases (GST) (Jancova et al. 2010; Zanger and Schwab 2013). These enzymes can transform a wide variety of substrates and one API can be metabolized by multiple different enzymes. However, for most APIs, there is only one or a few enzymes which contribute significantly to their metabolism, and there are some key enzymes

responsible for the biotransformation of most pharmaceuticals. Interspecific differences in the activity and selectivity of these key enzymes may determine the extent to which API's accumulate in different species, which in turn may lead to differences in sensitivity to pharmaceuticals (Perkins and Schlenk, 2000). Although the metabolism of pharmaceuticals in humans (and other mammals) is routinely studied as a part of the drug-development process, studies assessing biotransformation in fish are scarce and their capability to metabolize pharmaceuticals is less understood.

4.2 Bioaccumulation models

The bioaccumulation potential of a compound can be assessed in several ways by using different empirical measurements and mathematical models and are commonly described with bioaccumulation factors (BAF) and bioconcentration factors (BCF). The BCF for fish is referred to as the ratio between a chemical concentration in fish and a chemical concentration in water and expressed in l/kg (OECD 2012). BCF describes bioconcentration, so only non-dietary uptake of a compound is studied. This means that *in vivo* BCF studies are generally performed in laboratory settings, whereas field studies, where dietary uptake cannot be controlled, yield BAF values. Field studies give insight into the environmental fate and actual exposure and accumulation of a compound in wild fish. However, field conditions are not suitable for studying uptake and elimination kinetics in more detail, because the results are influenced by too many uncontrollable variables. In laboratory settings the results become more reproducible and are therefore better suited for classification and comparison purposes.

In *in vivo* studies, where fish are exposed to water containing a contaminant, a BCF value can be calculated at any given timepoint (OECD 2012). This means that the BCF is not set but may vary depending on exposure duration as well as other variables, such as contaminant concentration (Arnot and Gobas 2006). To produce reliable and comparable results, standardised tests have been made. It is stated in the 'Guideline on the environmental risk assessment of medicinal products for human use' that the bioaccumulation potential is to be evaluated experimentally if trigger values ($\log K_{ow} > 4.5$ or $\log K_{ow} \geq 3$ and PEC ≥ 0.01 µg/l) are met (European Medicines Agency 2006). This

is done by determining BCF in fish with an aqueous exposure test according to the Organisation for Economic Co-operation and Development (OECD) Test Guideline 305 'Bioaccumulation in Fish: Aqueous and Dietary Exposure' (OECD 2012). When performed in a standardised manner, *in vivo* bioconcentration tests give reliable and accurate information that can be used in the evaluation of a chemical's bioaccumulation potential, accounting for all pharmacokinetic properties including biotransformation. However, *in vivo* studies like the ones described in OECD Test Guideline 305 are highly expensive, time-consuming and require the use of a significant number of animals. A typical test takes over a month to complete and requires roughly 100 fish (OECD 2012; Burden et al 2014). Because of this, *in vivo* derived BCF values are scarcely available, and bioaccumulation assessments rely on computational models (Miller et al. 2019).

The most common computational models used to predict bioaccumulation in aquatic species in regulatory applications are quantitative structure-activity relationships (QSAR) models based on a correlation between BCF and $\log K_{ow}$ (Pavan et al. 2006). In these models, often a single equation explaining the relationship between $\log K_{ow}$ and measured BCF for a set of compounds is created and used to predict BCF for other chemicals. Models created in this way are highly influenced by the set of compounds and experimental BCF data used in the development of the model and are therefore not universally applicable. This is especially true if the compounds used to build the model are not closely related to the compounds for which the model is applied. Because of the large variety of physicochemical properties of pharmaceuticals, no single model can be used to reliably predict the BCF value for all of them. Furthermore, these models do not account for biotransformation and other pharmacokinetic parameters that have been shown to influence bioaccumulation.

A promising approach to overcome the challenge of producing reliable BCF estimates for compounds not already covered by existing databases, without the need for extensive *in vivo* testing, is to incorporate *in vitro* biotransformation data into computational models (Nichols et al. 2006; Cowan-Ellsberry et al. 2008). This way, relevant information directly concerning the test compound and organism of interest is used to produce refined BCF predictions. Transferring the focus to these types of methods is supported by the fact that i*n vitro* assays and *in silico* extrapolation tools are already routinely used in preclinical drug development to characterize clearance rates and metabolic pathways of

drug candidates in mammals (Ekins et al. 2000). In 2018 OECD published a set of guidelines describing the determination of *in vitro* intrinsic hepatic clearance in fish, including examples on how this data can be used to predict BCFs, with the aim to make such efforts standardized (OECD 2018a; OECD 2018b). However, in order to use these methods for evaluation of the bioaccumulation potential of pharmaceuticals in fish, the computational models need to be optimized to cover a wider range of compounds. Currently the main fallback is the lack of research on both *in vitro* biotransformation and *in vivo* bioconcentration on ionizable compounds. In this thesis, a set of preliminary assays was performed to evaluate the feasibility of the OECD Test Guideline 319B: Determination of *in vitro* intrinsic clearance using rainbow trout liver S9 subcellular fraction (RT-S9) for producing *in vitro* data on biotransformation of selected pharmaceuticals in fish.

5 AIM OF STUDY

The primary aim of this study was to assess whether selected pharmaceuticals, that are well metabolized in humans through key biotransformation pathways, are metabolized by rainbow trout liver enzymes *in vitro* under physiologically relevant conditions (11°C, pH 7.8). Using this new data and comparing it to previous studies, another aim was looking for patterns between the metabolism in humans and rainbow trout. A tertiary aim was to produce fish *in vitro* intrinsic clearance data, that could potentially be used as input in computational models to predict BCF, using a rainbow trout liver S9 substrate depletion assay. In order to produce reliable results, a large portion of the study was dedicated to validating the analytical method.

6 MATERIALS AND METHODS

6.1 Chemicals

Chemicals used in this study are listed in Appendix 1, along with purchase and available purity information. The same potassium phosphate buffer (7.8 ± 0.1, 100mM) was used throughout the study. Test compounds were selected based on environmental relevance and elimination pathway. All the compounds entered the market before 2006 and therefore predate the mandatory environmental risk assessment. They have been found in quantitative amounts in the environment and their main route of elimination in human is through metabolism (UNESCO and HELCOM 2017; DrugBank 2020, www.drugbank.ca). The test compounds are substrates to a range of different enzymes, and the main enzymes responsible for the initial step of metabolism of each API are listed in Table 2. Diclofenac was used as a reference compound based on recommendations of the OECD Test Guideline 319B (OECD 2018b). Structures of the test compounds are show in Figure 3.

Table 2. Test compounds and relevant pharmacological and chemical properties (Mano et al. 2007; Zanger and Schwab 2013; Wójcikowski et al. 2014; DrugBank 2020, www.drugbank.ca; CompTox Chemistry Dashboard 2021, https://comptox.epa.gov).

API	Classification	$\log K_{ow}$ EPI Suite	pKa	Primary routes of metabolism in human
Diclofenac	NSAID	4.0	4.0	CYP2C9
Gemfibrozil	antihyperlipidemic	4.8	4.4	UGT2B7
Haloperidol	antipsychotic	4.2	8.0	CYP3A4
Levomepromazine	antipsychotic	5.1	9.4	CYP1A2, CYP3A4
Levonorgestrel	hormonal	3.5	17.9	CYP3A4
Risperidone	antipsychotic	4.7	8.8	CYP2D6
Sertraline	antidepressant	5.3	9.9	CYP2B6, CYP3A4
Verapamil	antiarrhythmic	4.8	9.7	CYP3A4

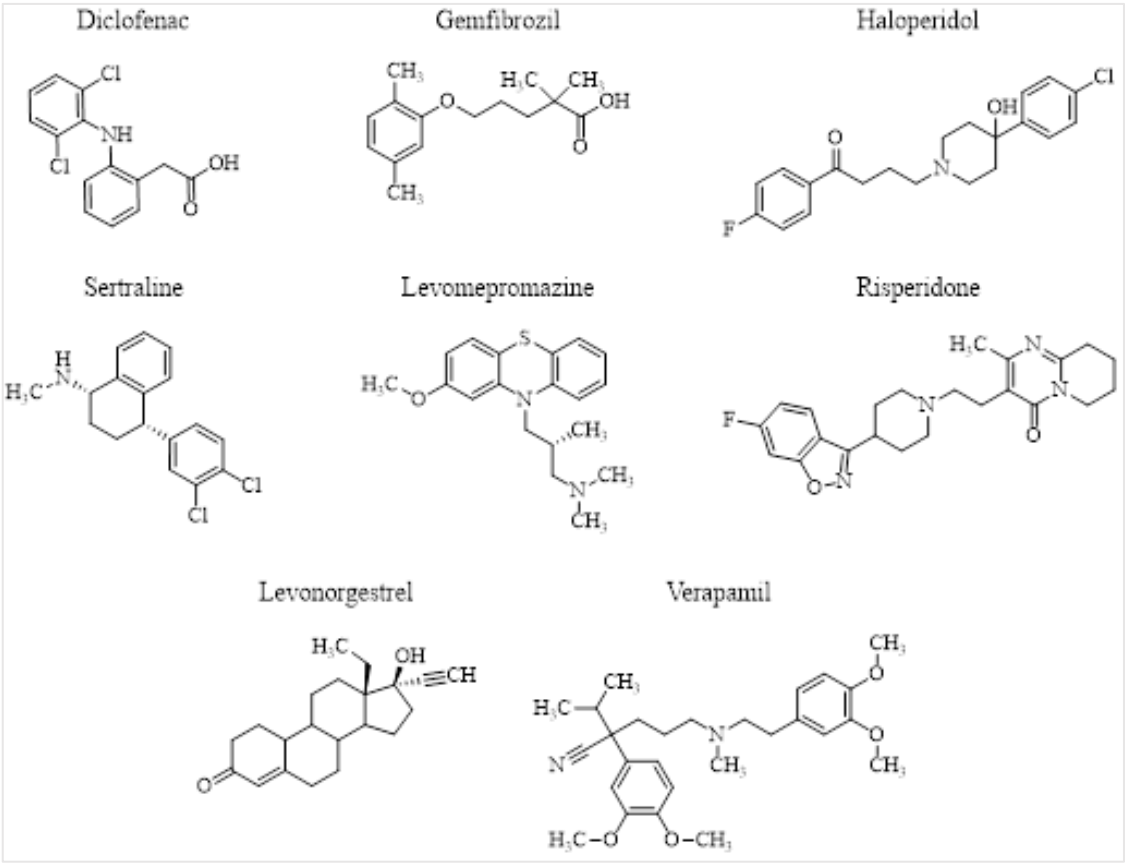

Figure 3. Chemical structures of the test compounds. Verapamil is a mixture of two enantiomers.

6.2 Enzyme source

Rainbow trout liver S9 subcellular fraction (RT-S9) was chosen for the *in vitro* system based on the OECD Test Guideline 319B. RT-S9 is relatively easy to use, allows for flexible incubation conditions and contains enzymes responsible for both phase I (CYP) and phase II (e.g. UGT, SULT, GST) metabolism (Mohutsky et al. 2008). As liver S9 subcellular fractions are derived from liver tissue homogenate, the enzymes are easily accessible compared to using intact liver cells and the biotransformation rate is therefore not influenced by cell membrane permeability. This way the baseline biotransformation ability of the microsomal fraction can be measured.

Active RT-S9 were purchased from PRIMACYT Cell Culture Technology GmbH. Product information and characterization provided by the supplier can be found in Appendix 2. For practical reasons a larger amount of the purchased RT-S9 was thawed on ice, divided into 50 µl aliquots, refrozen and finally stored at -80 °C until use. Enzymatically inactive RT-S9 was prepared by heating active RT-S9 in a 100 °C water bath for 15 minutes and stored at -20 °C until use.

6.3 *In vitro* metabolism assays: general incubation protocol

In vitro metabolism assays were performed as single vial incubation tests according to the OECD Test Guideline 319B. The principle of the assay is to examine substrate-depletion in the test system within a given time frame by measuring API concentration at different time points. In this study two kinds of incubation set-ups were used, consisting of different incubation and sampling volumes, sampling points and start concentrations (details in section 6.4 and 6.5). In both set-ups the incubation matrix consisted of RT-S9 at a concentration of 1 mg/ml and potassium phosphate buffer with added cofactors and alamethicin to support the biotransformation processes (Table 3). Alamethicin working solution was prepared by mixing 25 µl of 5 µg/ml alamethicin in ethanol with 475 µl of potassium phosphate buffer. Because of this, the final incubation mixture also contains 0.5% ethanol.

Table 3. Composition of the incubation matrix.

Reagent	Concentration in incubation matrix (1000 µl)
Potassium phosphate buffer (pH 7.8 ± 0.1, 100 mM)	-
RT-S9 fraction	1 mg/ml
Alamethicin	25 µg/ml
β-Nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt hydrate (NADPH)	2 mM
Uridine-5'-diphosphoglucuronic acid trisodium salt (UDPGA)	2 mM
Glutathione (GSH)	5 mM
3'-phosphoadenosine 5'-phosphosulfate (PAPS)	0.1 mM

RT-S9, potassium phosphate buffer and alamethicin were mixed in a 1.5 ml Eppendorf tube and pre-incubated on ice for 15 min before cofactors were added. Incubation vials were then moved to a 11°C cooling dry bath (Thermo Scientific). The reactions were initiated by adding a solution containing API to each incubation vial. Table 4 shows the diluting solvents used to prepare these solutions from the stocks, as well as the resulting final amount of DMSO/MeOH in the incubation mixture. Sampling at different time points was performed by removing an aliquot from the incubation vial and mixing it thoroughly with an equal volume of ice-cold acetonitrile (ACN) to denature the enzymes and stop the reaction. The samples were left on ice for at least 30 min, before centrifugation (10-15 min at 11800-13000 rpm). The supernatant was extracted and analysed by HPLC/UV on the same day.

Table 4. API solutions and the final amount of DMSO/MeOH in the incubation mixture. The concentration of API was 5µM for primary incubations and 1 µM for incubations for determining of *in vitro* intrinsic clearance.

API	Diluting solvent	The amount of DMSO/MeOH in final incubation mixture
Primary incubations		
Gemfibrozil	DMSO	1% (DMSO)
Haloperidol	DMSO	1% (DMSO)
Levomepromazine	DMSO	1% (DMSO)
Levonorgestrel	DMSO	1% (DMSO)
Risperidone	Potassium phosphate buffer (pH 7.8 ± 0.1, 100 mM)	<< 0.1% (MeOH)
Sertraline	DMSO	1% (DMSO)
Verapamil	MeOH	1% (MeOH)
Incubations for determining *in vitro* intrinsic clearance		
Levomepromazine	DMSO	0.5% (DMSO)
Diclofenac	Potassium phosphate buffer (pH 7.8 ± 0.1, 100 mM)	<< 0.1% (DMSO)

6.4 Primary incubations

Primary incubations were performed to get a preliminary insight on whether the test compounds are metabolized, and whether there would be any analytical challenges in need of attention. This was done for gemfibrozil, haloperidol, levomepromazine, levonorgestrel, risperidone, sertraline, and verapamil. Incubations were performed as duplicates for each test compound, carried out with negative controls containing heat inactivated RT-S9. A total of 1000 µl of incubation matrix was prepared and 200 µl was distributed into each incubation vial. Reactions were initiated by adding 2 µl of API solution to the incubation matrix to get an initial API concentration of 5 µM. Sample aliquots of 50 µl were collected at three timepoints (2, 60, and 180 min) and analysed by HPLC-UV.

6.5 Determination of *in vitro* intrinsic clearance in accordance to OECD 319B

Incubations to determine *in vitro* intrinsic clearance ($CL_{int,\ in\ vitro}$) were performed for diclofenac and levomepromazine. For both of these compounds, two independent runs on different days were performed with the previously mentioned negative controls. Additionally, diclofenac was incubated alongside levomepromazine as an external positive control. Reactions were initiated by adding 5 µl of API solution to 1000 µl of incubation matrix to get an initial API concentration of 1 µM. Samples of 100 µl were collected at nine different timepoints (2, 10, 20, 30, 60, 90, 120, 150, and 180 min).

6.6 Analytical method

Samples were analysed by HPLC-UV/Vis using an Agilent 1100 Series HPLC system equipped with a UV/Vis spectrophotometer and a 4.6 mm x 100 mm, Bonus-RP (C18) column (2.7µm, 120Å, Agilent Technologies). As eluents, 0.1% formic acid in deionized water (Milli-Q®) and 0.1% formic acid in methanol were used. Eluent gradients for each compound and their retention times are shown in Table 5. An injection volume of 50 µl

(30 µl for gemfibrozil) and a flow rate of 1 ml/min was used. The column temperature was set to 40 °C. Wavelengths used for detection and quantitation of the test compounds can also be found in Table 5.

Table 5. Eluent gradients for HPLC and detection wavelength. B = 0.1% formic acid in methanol, λ = wavelength.

Test compound (retention time)	Gradient		λ (nm)	Test compound	Gradient		λ (nm)
	Time (min)	B%			Time (min)	B%	
Diclofenac (5.1 min)	0.0	30	285	Levonorgestrel (5.3 min)	0.0	30	240
	2.0	80			2.0	70	
	10.0	100			9.0	100	
	11.0	100			10.0	100	
	11.5	30			10.5	30	
	15.0	30			14.0	30	
Gemfibrozil (9.4 min)	0.0	30	275	Risperidone (5.7 min)	0.0	10	280
	10.0	100			8.0	40	
	12.0	100			10.0	100	
	12.5	30			10.5	100	
	15.0	30			11.0	10	
Haloperidol (5.7 min)	0.0	10	245	Sertraline (6.4 min)	0.0	10	205
	10.0	100			10.0	100	
	10.5	100			10.5	100	
	11.0	10			11.0	10	
	14.0	10			14.0	10	
Levomepromazine (8.7 min)	0.0	10	254	Verapamil (8.4 min)	0.0	10	205
	9.0	50			9.0	50	
	11.0	100			11.0	100	
	11.5	100			11.5	100	
	12.0	10			12	10	
	15.0	10			15	10	

6.7 Method validation

The chromatographic methods used in this study had been preliminarily optimized before this work and were validated in relevant parts in accordance with U.S. Food and Drug Administrations (FDA) guideline “Bioanalytical Method Validation – Guidance for Industry” with some modifications (Ollikainen E, unpublished data; FDA 2018).

Standards were prepared in a 1:1 mixture of potassium phosphate buffer and stopping solution (ACN) unless otherwise stated.

To assess linearity, a minimum of six standard solutions with concentrations ranging from 0.25 µM to 4 µM (0.1 µM to 4 µM for levomepromazine and diclofenac) were prepared and analysed for each API. Peak area values were plotted against the corresponding standard concentration and linear regression was performed to obtain calibration curves ($R^2 \geq 0.990$) for each analyte (Appendix 3).

To assess selectivity, blank samples containing a mixture of potassium phosphate buffer and ACN (1:1), were analysed for interference at the retention time of the analyte. To determine the effect of carryover, a concentrated sample of API followed by a blank sample was analysed. No significant interference or carryover was detected.

Accuracy and precision studies were performed with diclofenac and levomepromazine. Three independent sets with five replicates at four different concentrations (0.1 (LLOQ), 0.3, 0.75, and 2 µM) were prepared and analysed. The measured concentrations obtained by using the calibration curve were compared with the nominal concentrations. The mean absolute percentage error and the relative standard deviation for each concentration level were calculated and are included in Appendix 3. In the incubation studies, to ensure consistent performance of the analytical equipment, duplicates of the 0.3, 0.75, and 2 µM samples were included in all runs as quality control, with an acceptance criterion of ±15%.

Recovery from matrix studies for diclofenac and levomepromazine were also performed. Two sets of five replicates of the 0.3, 0.75, and 2 µM samples were prepared. One set in incubation matrix containing inactivated RT-S9 and the other in the potassium phosphate buffer/ACN mixture. Matrix samples were prepared and stored similarly to study samples before analysis. Recovery of the test compound was calculated at each concentration by comparing measured peak area values in matrix to that in potassium phosphate buffer/ACN mixture. Percentage of recovery for diclofenac was 106% (±3.0%) and for levomepromazine 85% (±5.9%).

6.8 Data analysis

Measured concentrations of studied APIs were divided by the nominal starting concentrations and log-transformed and plotted as a function of time. Linear regression was performed on data from incubations with active and inactivated RT-S9. The slopes from these regression lines were compared with each other to assess for significant difference (p-value < 0.05) using Student's t-test. In addition, the slopes for incubations with inactivated RT-S9 were compared to 'slope = 0' to make sure there was no significant deviation in the negative control.

In vitro intrinsic clearance ($CL_{int,\ in\ vitro}$) for diclofenac and levomepromazine was calculated according to Equation 1. The first-order depletion rate constant (k_e) was obtained by multiplying the slope term from the regression equations by -2.3 (Johanning et al 2012). To convert ml/h/mg protein to ml/h/g liver an extrapolation factor of 50 mg microsomal protein/g liver was used.

$$CL_{int,\ in\ vitro} = \left[\left(\frac{k_e\ [min^{-1}] * \text{volume of incubation system [ml]}}{\text{quantity protein [mg]}} \right) * 60 \right] \qquad (1)$$

7 RESULTS AND DISCUSSION

7.1 Primary incubations

The results from primary incubations are shown in Figure 4. These results cannot be used for quantitative analysis of depletion rates as only three time points were measured, and the method was not fully validated for these compounds (excluding levomepromazine**)**. However, they give a qualitative estimate to whether the compound is metabolized in

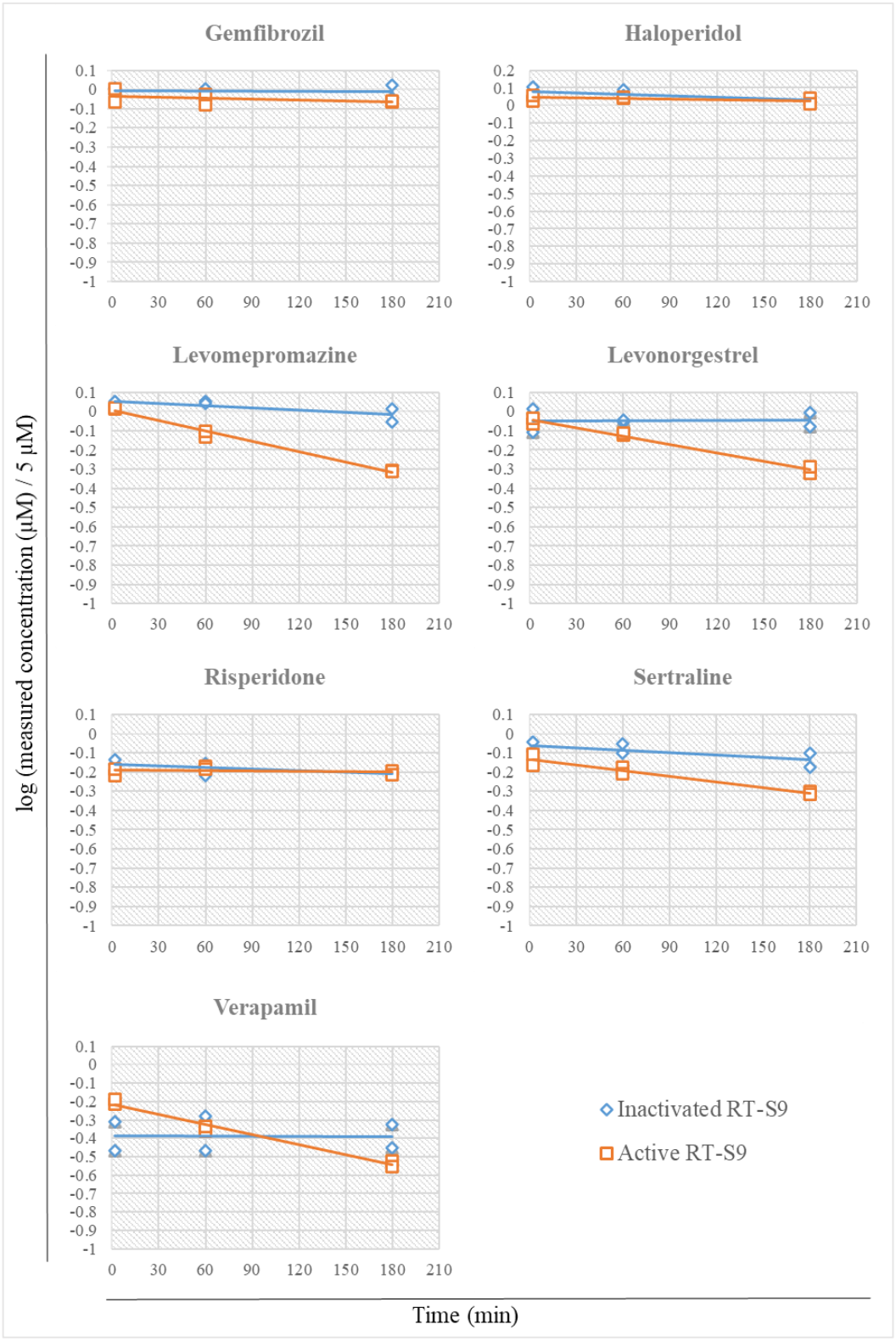

Figure 4. Biotransformation of selected pharmaceuticals by rainbow trout liver S9 fractions. Primary incubations for gemfibrozil, haloperidol, levomepromazine, levonorgestrel, risperidone, sertraline, and verapamil. Nominal API concentration was 5 µM.

rainbow trout liver microsomes. The observed substrate depletions cannot be attributed to a specific enzymatic process, as the RT-S9 fractions contain a variety of both phase I and phase II enzymes, and several enzymes may be responsible for the transformation of one substrate. It should be noted that the calibration curves were not made in matrix, but a 1:1 mixture of potassium phosphate buffer and ACN. Therefore, it is expected that the logarithmic value of the measured concentration divided by the nominal concentration at 2 min is slightly less than zero for both negative controls and samples with active RT-S9**,** unless the test compound is 100% recovered from matrix.

Levomepromazine, levonorgestrel and sertraline showed clear substrate depletion, with the slope for active RT-S9 being significantly different from the slope for inactivated RT-S9 (p-values: 0.000094, 0.0011 and 0.044, respectively), while gemfibrozil, haloperidol and risperidone showed no significant depletion over time. The negative control for levomepromazine and sertraline showed some, although not significant, loss of substrate during the assay**.** One possible cause for this could be abiotic degradation of the test compounds through hydrolysis or photolysis. The data from active incubation samples for verapamil also showed clear substrate depletion, while negative control samples showed no change in concentration over time**.** However, the measured concentration for the negative control is much lower than the nominal concentration. This could be due to adsorption onto surfaces or organic material in the incubation mixture, solubility problems, or human error. Issues with the negative controls should be addressed by aiming to optimize the incubation conditions prior to future testing.

Overall, the results from the primary incubations support previous observations where enzymatic activity between human and rainbow trout was compared by Connors et al. (2016). Here, levomepromazine, a human CYP1A2 substrate, is metabolized in rainbow trout *in vitro*. This was expected as fish are known to have CYP1A like activity and other CYP1A2 substrates, namely propranolol, show clear substrate depletion in RT-S9 systems. The CYP2D6 substate risperidone was not metabolized, similarly to previous findings where *in vitro* metabolism of CYP2D6 substrates methylphenidate and paroxetine was studied in RT-S9 (Connors et al. 2016). The metabolism of CYP3A4 substrates seems to be inconsistent, as levonorgestrel and sertraline were metabolized while haloperidol was not. Connors et al. (2016) made similar observations and speculated that either rainbow trout do not have an enzyme ortholog corresponding to

CYP3A4 and the substrates that are metabolized do so through other pathways, or that rainbow trout do express an enzyme with structural and functional similarity to human CYP3A4, but with some difference in substrate specificity**.** Either way, this inconsistency makes comparative prediction of metabolism between species difficult and supports the need of fish *in vitro* biotransformation studies and their use in bioaccumulation assessments.

A previously unreported finding in this study is the lack of metabolism of gemfibrozil in rainbow trout. In humans, gemfibrozil is glucuronidated mainly by UGT2B7 and not metabolized by CYP enzymes. Lack of substrate depletion in the assays suggests that rainbow trout lack UGT2B7 like activity, and that gemfibrozil, as well as the other compounds that did not display depletion by RT-S9, are more likely to accumulate in rainbow trout. However, further studies are needed to confirm these initial observations.

7.2 *In vitro* intrinsic clearance

The results of the substrate depletion assay for diclofenac can be seen in Figure 5. The $Cl_{int,\ in\ vitro}$ for diclofenac was calculated to be 6.2 ml/h/g liver (0.124 ml/h/mg protein). Similar clearance values (9.5 ml/h/g liver) have previously been reported for diclofenac and rainbow trout S9 fractions (Connors et al. 2013). There is some variability in the measurements within the first half hour of the incubation, and the slope of the curve is relatively shallow. It is stated in the OECD Test Guideline 319B that the slope should have a R^2 value ≥ 0.85, unless the compound shows slow biotransformation, in which case it is acceptable for the R^2 value to be below 0.85. Based on visual inspection and the depletion curve for diclofenac being significantly different from the negative control (p-value: 1.44×10^{-6}), the results can be considered reasonably reliable, despite the fact that the R^2 value is less (0.796) than the threshold value. This is supported by the results obtained when diclofenac was used as a positive control for levomepromazine (not shown), where the $Cl_{int,\ in\ vitro}$ and R^2 for diclofenac were calculated to be 7.5 ml/h/g liver and 0.895, respectively.

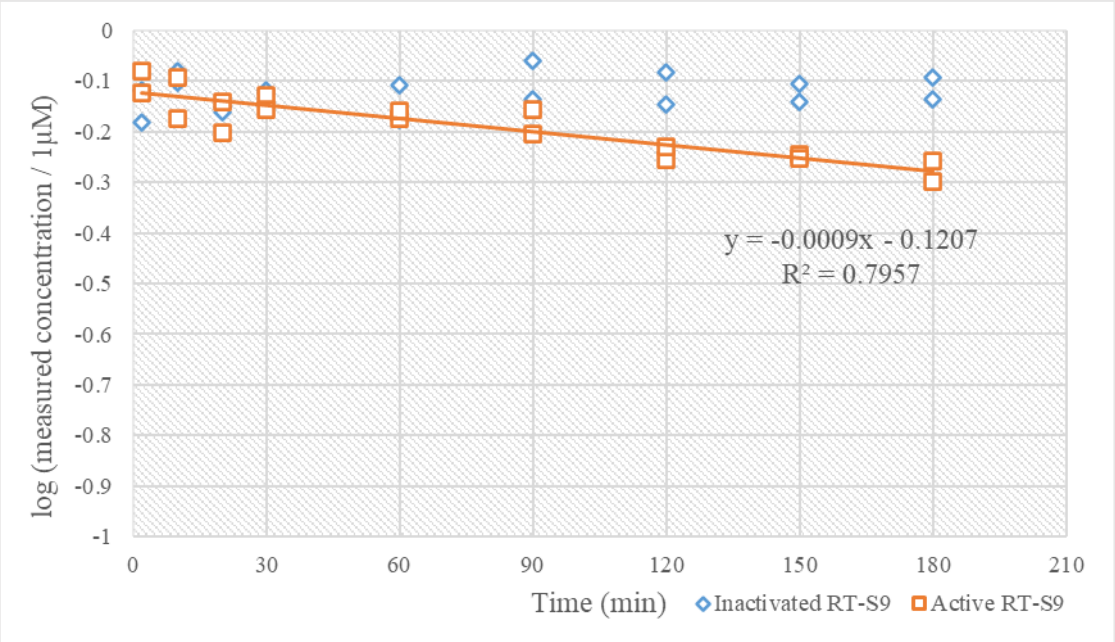

Figure 5. Biotransformation of diclofenac by RT liver S9 fractions. Nominal API concentration was 1 µM.

Results from the incubations performed to determine *in vitro* intrinsic clearance for levomepromazine can be seen in Figure 6. Levomepromazine is metabolised faster than diclofenac, with a $Cl_{int,\ in\ vitro}$ value of 26 ml/h/g liver (0.511 ml/h/mg protein). The calculated R^2 value is 0.958 and the slope is significantly different from the slope for the negative control (p-value: 4.28×10^{-15}). Levomepromazine concentration in the negative control does not change significantly over time. The logarithm of the concentration at 2 minutes is approximately -0.1 (Figure 6). This is in accordance with the results obtained from the recovery from matrix studies, which show the percent of recovery to be 85% (±5.9%).

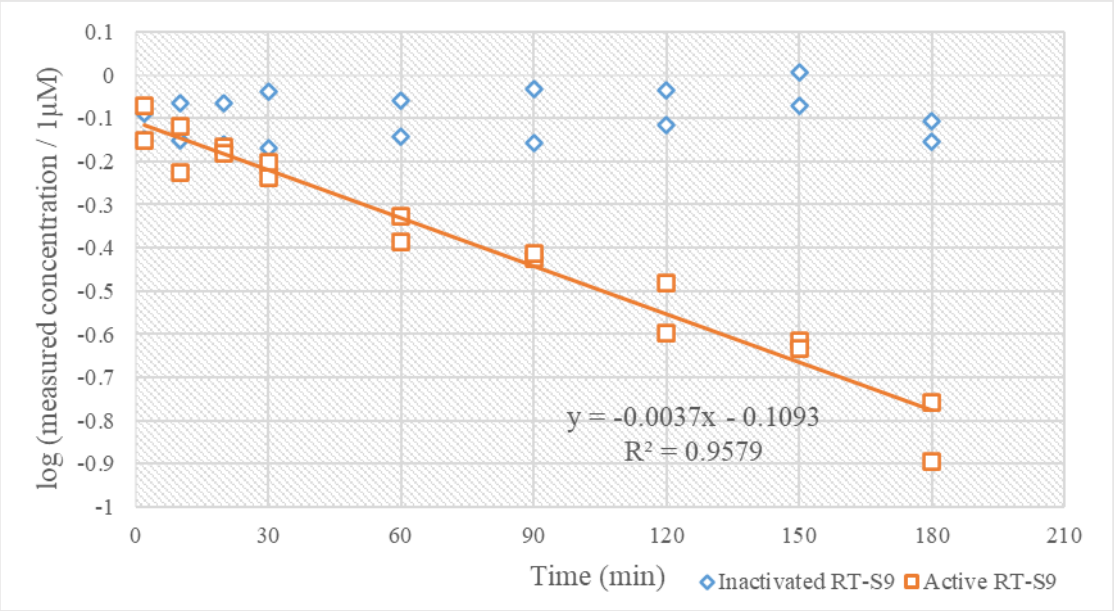

Figure 6. Biotransformation of levomepromazine by rainbow trout liver S9 fraction. Nominal API concentration was 1 µM.

7.3 Critical evaluation of the methodology

In this kind of substrate depletion study, there is a need to detect sub µM concentrations. Although the detection method used was generally sufficient for the reported compounds, UV absorbance might not be the most suitable alternative if the UV absorption of the analysed compound is not sufficient for detection, or if formed metabolites cannot be distinguished based on absorption. In this case, mass spectrometry would likely be a better alternative.

Generally thawing and refreezing of the RT-S9 fractions is not recommended as it might decrease the activity of the enzymes. However, such decline was not detected in this study. Plastic vials were used instead of glass vials because of incompatibilities with the available equipment, and the possibility of adsorption to plastic should be further investigated. Some adsorption tests were performed (not shown) and certain compounds (e.g. mirtazapine) were excluded based on extensive abiotic loss. Negative controls are

used to detect and correct for abiotic loss, but if the loss is significant it might lead to quantification issues.

Ideally, preliminary experiments, where RT-S9 and test compound concentrations are optimized, should be done prior to determination of $CL_{int,\ in\ vitro}$. Preliminary studies are made to ensure first-order kinetics as well as conditions that allow for the detection of substrate depletion and quantification of the substrate concentration throughout the whole incubation time. For example, if the enzyme concentration is too high, a test compound that is readily metabolized will deplete too rapidly, resulting in its concentration falling below the quantification limit before the required measurements can be made. Alternatively, a high substrate concentration can cause saturation of the enzymes, in which case the rate of metabolism plateaus and reaches its maximum for the specific concentration of enzymes. The rate of metabolism does not scale with increased substrate concentration after this point, leading to zero-order metabolism, which causes underestimation of $CL_{int,\ in\ vitro.}$ In the primary incubations performed in this study, a starting concentration of 5µM was used to assure reliable quantification. If this concentration has led to saturation of enzymes, optimization of the conditions might change the outcome for compounds that in this study are reported as not being metabolized. For diclofenac no preliminary tests were performed as suitable conditions have been previously characterised (Connors et al. 2013), and the optimisation and validation of the analytical method was done using the knowledge of the required quantification range. In the case of levomepromazine, generally recommended protein (1 mg/ml) and test compound concentrations (1µM) were used with no significant analytical problems. The logarithm of substrate depletion was linear and substrate depletion fell within the recommended range of 20% to 90%.

Some additional uncertainty also arises from the use of DMSO, which made up a maximum of 1% of the incubation mixture in this study (Table 4). DMSO has been shown to inhibit certain human CYP enzymes already at very low concentrations (Chauret et al. 1998). As the concentration needed for inhibition differs between metabolic pathways and the pathway responsible for an APIs elimination in fish liver systems is seldom known, the impact of DMSO cannot be fully predicted and therefore corrected for. In future tests, it may be preferrable to explore alternative solvents.

8 CONCLUSIONS AND FUTURE PROSPECTS

The results from the primary substrate depletion assays complement those of previous studies. Levomepromazine, a CYP1A2 substrate was readily metabolized by RT-S9 enzymes and CYP2D6 substrate risperidone was not. CYP3A4 substrates levonorgestrel and sertraline were metabolized while haloperidol was not. Additionally, a UGT2B7 substrate was also investigated, which had not been done in the previous studies. Gemfibrozil did not show depletion in the assays, leading to the initial observation that rainbow trout may lack UGT2B7 like activity. The apparent lack of *in vitro* metabolism of risperidone, haloperidol, and gemfibrozil combined with their lipophilic properties suggest that they are more likely to accumulate within rainbow trout, compared with the compounds that showed metabolic depletion, although repetitions and additional studies are needed to confirm this. In the studies for determination of *in vitro* intrinsic clearance levomepromazine displayed a higher clearance rate (26 ml/h/g liver) than diclofenac (6.2 ml/h/g liver). These intrinsic clearance values can potentially be used in computational models to produce BCF estimates.

The results of this study support the notion that a direct comparability between fish and human metabolism cannot be assumed, highlighting the need of fish *in vitro* biotransformation studies as a part of bioaccumulation assessments. Furthermore, these results could be used as a part of a wider data set to investigate whether biotransformation is sufficient to explain differences between calculated and experimental BCF values for ionizable compounds. However, this would require fish *in vivo* (whole body) BCF data, which is not currently available for most of the studied compounds. Even though this thesis has been focusing on single APIs, it is important to note that wild fish are not exposed to one substance at the time, but a mixture of chemicals. It would be interesting to study whether the metabolic ability of fish is altered, when exposed to a mixture of pharmaceuticals. As the baseline ability of the rainbow trout liver to metabolize selected pharmaceuticals was investigated in this study, it could be used as a reference point for such work.

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APPENDIX 1. Chemicals used, distributor, and available purity information. TRC = Toronto Research Chemicals

Purpose	Chemical	Distributor	Purity
Test compound/ Positive control	Diclofenac	Sigma-Aldrich	≥ 98%
Test compound	Gemfibrozil	Sigma-Aldrich	≥ 98%
Test compound	Haloperidol	TRC	-
Test compound	Levomepromazine	Sigma-Aldrich	-
Test compound	Levonorgestrel	TRC	-
Test compound	Risperidone	TRC	-
Test compound	Sertraline	TRC	-
Test compound	Verapamil	TRC	-
Cofactor	L-Glutathione reduced (GSH)	Sigma-Aldrich	≥ 98%
Cofactor	Uridine-5'-diphosphoglucuronic acid trisodium salt (UGDPA)	Sigma-Aldrich	≥ 98%
Cofactor	β-Nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt hydrate (NADPH)	Sigma-Aldrich	≥ 93%
Cofactor	3'-phosphoadenosine 5'-phosphosulfate (PAPS)	Sigma-Aldrich	≥ 60%
Pore-forming peptide	Alamethicin	A.G scientific	> 99%
Stopping solution	Acetonitrile (ACN)	Merk	> 99%
Solvent	Methanol	Merk	> 99%
Solvent	Dimethyl sulfoxide (DMSO)	Sigma-Aldrich	≥ 99.9%
Buffer	Potassium phosphate monobasic	Riedel-de-Haën	-
Buffer	Potassium phosphate diabasic	Amresco	-

APPENDIX 2: Product information and characterization of rainbow trout S9 subcellular fractions.

PRIMACYT
Cell Culture Technology

Subcellular fractions	
Lot Rainbow trout 180216 (Pool of 6)	Batch Release: June 08, 2018

Species: Rainbow trout (Oncorhynchus mykiss) Strain: Christophersen, Bornhoeved Supplier: Fish breeding Christophersen Acclimation temperature: 13.2 ± 1.3 °C Age: approx. 2 years	Number and gender of animals: 6 (3 female, 3 male) sexual immature All animals were kept under controlled housing conditions at Fraunhofer EMB in Lübeck.

Animal characteristics:

Donor	**1**	**2**	**3**	**4**	**5**	**6**
Gender	male			female		
Fish weight (g)	400	302	315	397	325	399
Liver weight (g)	5.82	3.95	3.88	3.72	4.94	3.93
Gonad weight (g)	0.09	0.08	0.10	0.53	0.46	0.37
GSI (gonad weight/fish weight)	0.02	0.03	0.03	0.13	0.14	0.09

GSI = Gonadosomatic index

Product Number	**Product Description**	**Amount**	**Protein content**
RTL-S9-180216	Rainbow trout S9 Fraction, mixed gender	0.5 ml	20 mg/ml

Enzyme assay results	
Assay	**Enzyme activity (nM/min)** **mean ± SD**
Phenacetin-O-deethylase	3.26 ± 0.58
Bupropion-hydroxylase	0.52 ± 0.05
Diclofenac 4'-hydroxylase	41.1 ± 5.8
Bufuralol 1'-hydroxylase	0.63 ± 0.10
Midazolam 1'-hydroxylase	1.95 ± 0.21
UDP-Glucuronosyltransferase	1,394.3 ± 230.8
Sulfotransferase	42.0 ± 2.2

Note: Activity assays were performed at PRIMACYT GmbH. The assays were conducted at 1 mg/ml protein in 0.1 M Phosphate buffer at 37 °C for 15 min (Phase I) and for 30 min (Phase II).

APPENDIX 3. Method validation results. For accuracy and precision studies, 3 sets of 5 repeats (n = 15) were performed at each concentration level.

API	Range (µM)	Slope equation for standard curve	R^2
Diclofenac	0.1-2	y = 29.795x + 0.5405	0.9993
Gemfibrozil	0.5-4	y = 3.3126x + 0.163	0.9907
Haloperidol	0.25-4	y = 30.895x + 0.0607	0.9999
Levomepromazine	0.1-4	y = 65.962x + 0.5872	0.9998
Levonorgestrel	0.25-4	y = 10.161x - 0.3864	0.9998
Risperidone	0.25-4	y = 30.489x + 8.4738	0.9761
Sertraline	0.25-4	y = 29.792x - 4.1938	0.9919
Verapamil	0.25-4	y = 165.06x + 5.0592	0.9940

	Diclofenac				**Levomepromazine**			
The lower limit of quantification (µM)	0.1				0.1			
Accuracy and Precision								
Concentrations (µM)	0.1	0.3	0.75	2	0.1	0.3	0.75	2
Mean absolute percentage error (%)	2.2	4.1	4.3	2.1	6.8	2.5	1.3	1.3
Relative standard deviation (%)	19.4	3.67	2.47	1.94	2.71	3.84	1.09	0.85
Recovery from matrix (%)	106 ±3.0				85 ±5.9			